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Effect of Nucleosome Assembly on Alkylation by a Dynamic Electrophile

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Abstract

Quinone methides are reactive electrophiles that are generated during metabolism of various drugs, natural products, and food additives. Their chemical properties and cellular effects have been described previously, and now their response to packaging DNA in a nucleosome core is described. A model bisquinone methide precursor (bisQMP) was selected based on its ability to form reversible adducts with guanine N7 that allow for their redistribution and transfer after quinone methide regeneration. Assembly of Widom's 601 DNA with the histone octamer of H2A, H2B, H3, and H4 from *Xenopus laevis* significantly suppressed alkylation of the DNA. This result is a function of DNA packaging since addition of the octamer without nucleosome reconstitution only mildly protected DNA from alkylation. The lack of competition between nucleophiles of DNA and the histones was consistent with the limited number of adducts formed by the histones as detected by tryptic digestion and ultraperformance liquid chromatography–mass spectrometry. Only three peptide adducts were observed after reaction with a monofunctional analogue of bisQMP, and only two peptide adducts were observed after reaction with bisQMP. Histone reaction was also suppressed when reconstituted into the nucleosome core particle. However, bisQMP was capable of cross-linking the DNA and histones in moderate yields (~20%) that exceeded expectations derived from reaction of cisplatin, nitrogen mustards, and diepoxybutane. The core histones also demonstrated a protective function against dynamic alkylation by trapping the reactive quinone methide after its spontaneous regeneration from DNA adducts.

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Supporting Information

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Supplementary experimental procedures, tables, figures (PDF)

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INTRODUCTION

Reactions between nucleic acids and metabolites, drugs, or toxic agents are often first described using well-defined model systems based on DNA free in solution. This approach is most expedient for characterizing the molecular details of lesion formation, but the results may not always reflect the nature of cellular DNA. Genomic DNA of Eukaryotes is wrapped around protein octamers containing two copies each of four histones (H2A, H2B, H3, and H4) to form nucleosome core particles (NCPs).^{1,2} The electrostatic environment, solvent accessibility, and duplex geometry of DNA are affected by NCP assembly, and hence, its reactivity has the potential to change relative to that for DNA free in solution.³ The impact of these differences is highly dependent on the reaction studied. For example, nucleosome assembly does not significantly affect the alkylation of guanine N7 in the major groove nor adenine N3 in the minor groove by the relatively small and neutral reagent dimethyl sulfate.⁴ For reactants larger than dimethyl sulfate, the influence of NCP is often observed.

Reaction of minor groove specific compounds such as mitomycin C,⁵ duocarmycin B₂,⁶ and benzo[a]pyrene diol epoxide^{7,8} is suppressed in nucleosomal versus free DNA. Such a response is typical but not universal since duocarmycin SA and yatakemycin also target the minor groove but remain unaffected by NCP formation.⁹ Reaction of the major groove is often suppressed as well. Alkylation of guanine N7 by a series of nitrogen mustards⁵ and a dichloro derivative of aflatoxin B₁¹⁰ was generally suppressed by nucleosome assembly. Perhaps surprisingly, the overall sequence selectivity of reaction remained relatively constant in both examples. When suppression is observed, the effects are usually greatest in the vicinity of the dyad axis where the DNA duplex is most distorted.^{5,7,11} The structural constraints within the NCP also typically limit binding of intercalators except for a region in which the helix is stretched to extend through the dyad axis.^{3,12} Of course, the histones surrounding the DNA also present nucleophiles that may compete with DNA for electrophilic intermediates as illustrated by reaction of 4-oxo-2-nonenal that derives from oxidation of cellular lipids.¹³ The histone nucleophiles similarly have the potential to react with crosslinking agents along with DNA to form protein–DNA adducts, although other cellular proteins typically outcompete histones in such reactions.^{14,15}

The relative reactivity of quinone methide intermediates (QM) with DNA associated with the NCP has yet to be investigated and is now the subject of our studies reported here. Such intermediates are highly electrophilic and often persist only transiently before reacting with strong nucleophiles.¹⁶ A variety of metabolic processes *in vivo* have the potential to generate numerous QMs. For example, the food preservative BHT and anticancer drug tamoxifen are transformed into their QM derivatives by enzyme catalyzed oxidation.^{17–20} In contrast, reduction is needed for generating the QM-like intermediate of mitomycin C that is responsible for DNA cross-linking,²¹ and hydrolysis is needed to initiate formation of QMs from certain NO-NSAIDs.^{22–24} Our laboratory began investigations on a model *ortho*-QM that forms reversible and irreversible adducts as a function of the nucleophilicity and leaving group ability of DNA functional groups (Scheme 1).²⁵ Adducts formed by cytosine N3 and adenine N1 dominate the initial product profile due to their high nucleophilicity. However, both adducts dissipate over time since these sites also behave as stable leaving groups and

release QM for further reaction. These results are evident for reaction of both nucleosides and duplex DNA.²⁶

Specificity of alkylation can alternatively be controlled by conjugating a QM to ligands selective for DNA that may, for example, target the major or minor grooves of selected sequences.^{27,28} A bisfunctional analogue conjugated to acridine (bisQMP, Scheme 1) was concurrently developed as an efficient cross-linking agent of DNA by alkylating guanine N7 in the major groove.² The strong leaving group ability of guanine N7 also supports the dynamic regeneration of QM intermediates to allow transfer of the cross-link between strands of DNA and along its double helical structure.^{30,31} How this conjugate responds to the presence of histones and the packaging of DNA in the NCP has now been characterized using the standard NCP model based on the Widom 601 DNA³² and core histones of *Xenopus laevis* generated by heterologous expression.^{33,34}

MATERIALS AND METHODS

General Materials.

Oligonucleotides (desalted) were obtained from IDT and gel purified prior to use. BisQMP was prepared as described previously,³¹ and monoQMP was a gift from Blessing Deeyaa. The Widom 601 DNA³² was constructed by enzymatically ligating oligonucleotide fragments (30–35 nt) and radiolabeled at a 5'-terminus with [³²P] as described previously (Figure S1).³⁴ The core histones (*Xenopus laevis*) were expressed in *Escherichia coli* and assembled *in vitro* into the native octamer under standard conditions.¹ T4 polynucleotide kinase, T4 DNA ligase, and proteinase K were obtained from New England Biolabs. Benzoylarginine and trypsin (powder, Bovine pancreas) were purchased from Sigma. γ -[³²P]-ATP was purchased from PerkinElmer. C₁₈-Sep Pak cartridges were purchased from Waters Corp. Salmon sperm DNA (10 mg/mL) was purchased from Invitrogen. Amicon Ultra centrifugal filters with a 10 000 molecular weight cutoff were purchased from Millipore.

General Procedures.

All reactions and digestions were conducted in siliconized tubes from Bio Plas Incorporated. Radiolabeled oligonucleotides were detected and quantified using a Typhoon 9400 phosphorimager equipped with ImageQuant TL software. Oligonucleotide concentrations were calculated from their absorption at 260 nm and extinction coefficients that were provided by the manufacturer. Histone concentration was determined directly by its absorbance at 276 nm.¹

Reconstitution of Nucleosome Core Particle (NCP).

Preparation of the NCP followed published procedures except the incubation temperature was reduced from 30 °C to ambient conditions.³⁴ The preassembled histone octamer (84 pmol) in 2 M NaCl (1 μ L) was added to a solution of salmon sperm DNA (84 pmol) and 5'-[³²P]-radiolabeled 601 DNA (~1 pmol) in 2 M NaCl, 10 mM HEPES pH 7.8, and 1 mM EDTA (10 μ L) with 1 mg/mL BSA. The mixture was then incubated for 1 h before serial dilution with nucleosome reconstitution buffer (10 mM HEPES pH 7.5, 1 mM EDTA and 1 mg/mL BSA). After the final dilution (total volume 224 μ L), a small aliquot (5 μ L) was

removed and analyzed by native gel electrophoresis (6%, acrylamide/bis(acrylamide), 59:1, $0.6 \times$ TBE) to determine the NCP reconstitution efficiency. The final solution was stored at 4 °C until use.

Treatment with BisQM.

5'-[32 P]-Radiolabeled 601 DNA (0.08 pmol) in the alternative presence and absence of the histone octamer (5.6 pmol) was added to a solution of salmon sperm DNA (5.6 pmol) in 10 mM MES, 10 mM NaF, 7.5 mM HEPES, 0.75 mM EDTA, 0.75 mg/mL BSA, and 67 mM NaCl at pH 7.0 (20 μ L). An equivalent concentration of reconstituted NCP formed by 5'-[32 P]-labeled 601 DNA (0.08 pmol) was supplemented with 10 mM MES pH 7.0 and 10 mM NaF to maintain similar solvent conditions. The indicated bisQMP in acetonitrile was then added to the three individual samples above along with sufficient acetonitrile to generate a constant 20% solution. Samples (20 μ L) were incubated at 4 °C for 24 h and quenched by freezing in liquid nitrogen. For the DNA analyses below, samples were treated with proteinase K (1.6 U) for 15 min just prior to quenching.

Piperidine Treatment of Alkylated DNA.

Frozen samples treated with bisQMP above were lyophilized and dissolved in 5% piperidine (10 μ L). The resulting solutions were heated for 30 min at 90 °C and lyophilized again. To remove residual piperidine, samples were again dissolved in water (30 μ L) and lyophilized in three consecutive repetitions. Samples were finally dissolved in water (10 μ L) and combined with bromophenol blue and xylene cyanol in formamide (10 μ L) for separation by 10% denaturing PAGE.

Protein Adducts Detected by Liquid Chromatography-Mass Spectrometry.

Equal concentrations (2.5 μ M) of the histone octamer and reconstituted NCP in 10 mM HEPES, 1 mM EDTA, 1 mg/mL BSA, and 89 mM NaCl were individually combined with 10 mM MES pH 7 and 10 mM NaF. The resulting mixtures were treated alternatively with monoQMP or bisQMP (500 μ M) in acetonitrile to a final volume of 20 μ L with 20% acetonitrile and incubated at 4 °C for 24 h. Samples of NCP were subsequently treated with benzonase (250 U) for 30 min at 37 °C. All samples were then lyophilized, resuspended in 3.6 M guanidine-HCl, 50 mM Tris-HCl pH 7.2, and 2 mM DTT to a final volume of 20 μ L and heated (65 °C) for 45 min to denature the protein. Samples were subsequently diluted with 1 mM CaCl_2 and 50 mM Tris-HCl pH 7.2 to a final volume of 75 μ L. Trypsin (111 mM potassium phosphate pH 7.4) was added to generate a trypsin to protein ratio of 1:20, and the samples were incubated at 37 °C for 24 h. The resulting peptides were desalted by elution through a C₁₈ - Sep Pak with 50% aq. CH_3CN and 0.1% formic acid and analyzed by UPLC-MS on an Acquity UPLC H-Class/Xevo G2 QToF equipped with a 2.1 mm $\times$ 100 mm HSST3-C18 column (1.7 μ m pore size). Sample separation was accomplished with an initial 5% aq. acetonitrile solution with 0.1% formic acid (1 min) followed by a linear gradient to 40% CH_3CN over 36 min and then to 95% CH_3CN over 3 min with a flow rate of 0.3 mL/min. Mass spectra were acquired in the ESI positive ion mode with MS^E using a capillary voltage of 3 kV, a sample cone voltage of 30 V, and an extraction cone voltage of 4 V. The cone and desolvation gas flow was 30 L/h and 800 L/h, respectively. Desolvation and source temperatures were 400 and 150 °C, respectively. The scan acquisition rate was 10 Hz

for m/z of 100–3000. MassLynx and BioPharmLlynx software were used to analyze the resulting data.

Reconstitution of NCP with Alkylated DNA.

5'-[^{32}P -Radio-labeled 601 DNA (1 pmol) and salmon sperm DNA (84 pmol) were incubated with bisQMP (250 μM) in 10 mM MES pH 7 and 10 mM NaF for 24 h at room temperature. Excess bisQMP and its low molecular weight products were removed from the DNA with a BioRad P6 spin column prewashed with water (1000 g, 4 min). The eluant was lyophilized, resuspended in 5 μL of H_2O , and used for nucleosome reconstitution as described above for the parent 601 DNA.

Reconstitution of NCP with Alkylated Histone Octamer.

BisQMP (500 μM) was incubated with the histone octamer (84 pmol) in 2 M NaCl, 10 mM MES pH 7.0, and 10 mM NaF for 24 h at 4 $^{\circ}\text{C}$. Removal of excess bisQMP and NCP reconstitution were then performed in analogy to that described above for the alkylated DNA.

Distribution of Alkylated DNA after NCP Reconstitution.

Solutions of NCP reconstituted with alkylated DNA described above were concentrated to 30 μL using a 0.5 mL Amicon Ultra centrifugal filter with a molecular weight cutoff of 10 000 Da (4 $^{\circ}\text{C}$). DNA associated with the reconstituted NCP and the remaining free DNA were separated by native PAGE (6%, acrylamide/bis(acrylamide), 59:1, 0.6 $\times$ TBE) using a running buffer of 0.2 $\times$ TBE and 200 V (1 h, 4 $^{\circ}\text{C}$). The DNA species were detected by phosphorimager and extracted from the gels by excision, maceration, and finally immersion in 2 mL of elution buffer (0.2 M NaCl, 1 mM EDTA) overnight at 4 $^{\circ}\text{C}$. Solid material was removed by passage through a Bio-Rad Poly-Prep column and the eluant was lyophilized. The isolated DNA was incubated with proteinase K (1.6 U) for 15 min, heated with piperidine for cleavage at sites of guanine N7 alkylation, and analyzed as described above.

QM Transfer From DNA to Histone Octamer.

The 601 DNA (30 nM) and salmon sperm DNA (2.47 μM) in 10 mM MES pH 7 and 10 mM NaF were treated alternatively with monoQMP and bisQMP (500 μM) for 24 h at room temperature. Excess QM and its low molecular weight products were removed from DNA with a BioRad P6 spin column prewashed with water (1000 g, 4 min). Histone octamer (2.5 μM) in 10 mM HEPES pH 7.8, 1 mM EDTA, 1 mg/mL BSA, and 89 mM NaCl was added to the resulting DNA and incubated at 4 $^{\circ}\text{C}$ for 24 h. Samples were then digested with benzonase and trypsin and analyzed as described above to identify peptide adducts by UPLC–MS.

RESULTS

Effect of Nucleosome Assembly on DNA Alkylation by BisQM.

An NCP generated from Widom's 601 sequence (Figure S1) and *Xenopus laevis* histones H2A, H2B, H3, and H4 expressed in *E. coli* was chosen to provide a homogeneous target for

QM reaction. This sequence was originally identified for its ability to form a highly stable NCP with uniform positional and rotational orientation.³² Heterologous expression of the individual histones avoids potential contamination by natural histone variants and epigenetic modifications that are present in NCP isolated from endogeneous sources. The effect of DNA packaging on its reactivity with a QM was first compared by treating DNA free in solution and assembled in NCPs for 24 h with bisQMP ranging from 0 to 500 μ M. BisQMP represents the stable precursor to its reactive QM intermediate that forms spontaneously after removal of the silyl protecting group with fluoride (Scheme 1).²⁹ Reaction samples were subsequently treated with piperidine to induce fragmentation at sites of guanine N7 alkylation, the primary target of bisQMP (Figure 1).²⁹ These conditions were sufficient to consume the free DNA almost completely in the presence of 250 μ M of bisQMP. In stark contrast, only 10% of the DNA in the NCP sample was consumed in the presence of 500 μ M of bisQMP after 24 h. If this minimal reactivity was due to modification of DNA dissociated from the NCP, then suppression by the NCP is even greater than that indicated by Figure 1. Overall, the profile of guanine N7 alkylation was not significantly different in these two targets since the relative distribution of fragments remains similar. Equivalent profiles are also maintained over the time of reaction prior to consumption of the parent DNA (Figures S2).

The reactivity of DNA was also examined in the competing presence of the histone octamer when not assembled into a NCP to distinguish the effects of DNA packaging from the added concentration of competing nucleophiles provided by the histones. Full consumption of the DNA after incubation with 500 μ M of bisQM for 24 h was not detected in the presence of the octamer, but the yield of dGN7 alkylation (80%) was still much more similar to that observed with the unpackaged DNA than that with the NCP (Figure 1). Again, the distribution of products appears similar in all three samples. Nonspecific association of the DNA and octamer is possible in the absence of NCP reconstitution, but this did not dramatically influence the reaction. Sufficient QM remains in the presence of the histones for extensive alkylation of the DNA. Thus, the packaging of DNA to form the NCP, rather than the general presence of the histones, is responsible for the suppression of DNA alkylation by the bisQM.

DNA–Protein Cross-Linking by BisQM.

The bifunctional nature of bisQMP was originally designed for crosslinking strands of DNA,²⁹ but it also has the potential to crosslink any reactive pair of nucleophiles. The extent of DNA–protein cross-linking within the NCP was assessed by the sensitivity of reaction products to proteinase K. NCP was incubated with fluoride and the indicated concentrations of bisQMP to generate the reactive intermediate bisQM (Figure 2). Reaction was quenched after 24 h and products were separated by SDS-PAGE. Histone-DNA cross-links were evident from the accumulation of products with low electrophoretic mobility and the heterogeneity in mobility suggests an equivalent heterogeneity in product composition and connectivity. Minimal cross-linking was observed after treatment with 50 μ M of bisQM, but the yield increased to ~20% after treatment with 500 μ M of bisQM. The presence of protein in the continuum of low mobility products was confirmed by their loss after subsequent digestion with proteinase K (Figures 2 and S9).

Alkylation of Histone Octamer by MonoQM.

The monofunctional QM (monoQM, Scheme 1) was used initially to identify the potential sites of histone alkylation without the complications introduced by the analogous bifunctional bisQM. Additionally, investigations began with a focus on the histone octamer rather than the NCP to identify the maximum number of possible sites of reaction between the histone side chains and the model QM. Limited proteolysis of the octamer and subsequent UPLC–MS analysis provided good coverage of the histones (Figures 3A and S3). Equivalent analysis of the octamer after reaction with monoQM (500 μ M) for 24 h yielded six new species apparent in the UPLC chromatogram (Figure 3A, Table 1). The species indicated as the QM-H₂O adduct formed by quenching the monoQM by solvent. Two other species observed in the presence of monoQM did not correspond to histone adducts (marked with “0”, Figure 3A). The remaining species numbered I–III (Table 1) generated parent ions corresponding to the combined mass of their amino acid sequence and monoQM (Figures 3C and S4A,B). Two of these (I, III) are generated by alkylation of TESSK and IAGEASR that are unique to histones H2A and H2B, respectively. The third (II) contains RR and may be derived from multiple sites in histones H2B, H3, and H4. The presence of secondary ions from the corresponding MS² also identified the monoQM and a characteristic fragment of its 9-aminoacridine component.

Alkylation of Histone Octamer by BisQM.

The histone octamer was treated analogously with bisQMP to identify its reaction sites as well. This bifunctional precursor is capable of generating two QM intermediates in tandem for reaction with two protein nucleophiles or alternatively with one protein nucleophile and water. Only two bisQM adducts containing peptides were observed after tryptic digest and neither exhibited [M + H]⁺ that fully described reaction at each electrophilic site (Figure 3B). Data reveal coupling to only individual peptides (IV and V, Table 1). Whatever reacted at the second benzylic position was likely lost during the ionization process of MS. The [M + H]⁺ of products IV and V suggest adducts of SAK and DIQLAR from histones H2A and H3, respectively, and the MS² of these products generate the expected [M + H]⁺ for fragments of the bisQM (Figures 3D and S4C). Interestingly, the sites of reaction within the histones are not identical for monoQM and bisQM.

Alkylation of Histones in NCP.

The NCP was treated with monoQM (500 μ M) for 24 h, digested with trypsin, and analyzed by UPLC–MS as described above to measure the effect of nucleosome assembly on protein alkylation. Only the adduct formed by TESSK of histone H2A was observed under these conditions (Figure S5A, Table 1, entry I). Similar treatment of the NCP with bisQM yielded no identifiable protein adducts. Thus, assembly of the NCP suppresses reaction of the QMs with both the histones and, as presented above, the DNA.

Nucleosome Assembly after Exposure to BisQM.

Nucleosome assembly is a dynamic process *in vivo* and has the potential to expose both DNA and histones to conditions that would allow for their modification by electrophiles. To test whether bisQM-treated DNA affected the formation of NCP, 601 DNA free in solution

was treated with bisQM (250 μ M, 24 h) and excess bisQM was then removed via gel filtration. Approximately 80% of this DNA exhibited a slightly retarded migration during native PAGE relative to untreated DNA (Figure 4A). The alkylated DNA was then subjected to standard conditions for NCP reconstitution with the histone octamer. Assembly of the NCP under these conditions was limited to approximately 25% of the 601 DNA, and the remaining 75% migrated as free DNA (Figure 4A). In contrast, the NCP is typically formed in greater than 95% yield based on the 601 DNA (Figure S6A). Similarly high yields are also detected for reconstitution using DNA that has been incubated with bisQMP for 24 h in the absence of fluoride that is required for bisQM formation (Figure S6B). Thus, covalent modification of DNA by bisQM rather than intercalation by the acridine of bisQMP is likely responsible for the observed suppression of NCP formation.

The alkylated DNA used to reconstitute NCP was also separated by native PAGE to determine if reconstitution was tolerant to modification in certain regions but intolerant at other regions. The fractions of DNA that assembled into the NCP and the DNA remaining unassembled were independently treated with piperidine to profile their modifications at guanine N7 (Figure S7). Not surprisingly, a greater extent of modification was observed in the fraction of DNA that remained unassembled, but the relative profile of alkylation sites in the assembled and unassembled DNA remained similar. This suggests a general destabilization of NCP formation by alkylation of DNA with bisQM and no preferential tolerance of modification at specific positions along the NCP was apparent. A significantly lower yield of guanine N7 alkylation is detected for samples incubated over more than 8 h during reconstitution and gel separation relative to samples treated directly with piperidine (Figure S7). This difference is likely a consequence of the reversibility of guanine N7 alkylation that allows for transfer and quenching of the bisQM adduct with water during NCP reconstitution.^{30,35}

In a complementary study, NCP assembly was measured after reconstituting the parent 601 DNA with histone octamer that had been treated with bisQM (500 μ M, 24 h). NCP formation was not impeded under these conditions (Figure 4B) and yields compared favorably to those using unmodified components (Figure S6A). Hence, NCP assembly is relatively insensitive to the minimal alkylation of histones observed with bisQM (Figure 3B, Table 1).

QM Transfer from DNA to Histone Octamer.

The reversibility of guanine N7 alkylation by QMs had alternatively been observed by transfer of the electrophile from nucleobase adducts to water, trapping agents, and competing nucleobases.^{25,30,35} The histone octamer also has the potential to trap QM released after spontaneous regeneration from its adducts of guanine N7. This possibility was first tested with 601 DNA treated with monoQM (500 μ M) for 24 h and then incubated with the histone octamer for an additional 24 h without reconstitution. The incubation period was chosen based on an equivalent time necessary for bisQM to complete an intra- to interstrand transfer of DNA cross-linking.³¹ After treatment, the octamer was digested by trypsin, and the resulting peptides were analyzed by UPLC–MS as described above (Scheme 2). Under these conditions, the monoQM adducts of TESSK and IAGEASR of histones H2A and H2B

(Table 1, entry I, III), but not RR (Table 1, entry II) (Figure S8A), were detected. An equivalent analysis of bisQM transfer from DNA to histones was performed and revealed a similar activity. BisQM adducts of DNA were capable of transferring to SAK of histone H2A (Table 1, entry IV) but not DIQLAR of histone H3 (Table 1, entry V) (Figure S8B). As expected, both QMs were also quenched by water in competition with histone modification (Figure S8). The substantial release of QM during the reconstitution of NCP (Figure S7) prevented comparable studies to assess transfer of QM between DNA and histones within NCP, but their proximity can be expected to facilitate the process during exposure to a QM that reacts reversibly.

DISCUSSION

Alkylation of DNA by QM Is Suppressed by NCP Assembly.

Guanine N7 in the major groove of DNA is the primary site of alkylation by the QM-acridine conjugate and readily detected by strand scission after subsequent treatment with piperidine.²⁹ Reaction at this site is significantly suppressed by NCP assembly (Figure 1). The decrease of alkylation also appeared relatively uniform throughout the 601 DNA sequence suggesting that access of the QM conjugates to the major groove was not selectively blocked when this region is oriented toward the histone octamer. Similarly, the kinking and bending of DNA within NCP did not affect the specificity of bisQM reaction, although these perturbations are known to impair binding of certain DNA targeting drugs.³⁶ A general loss of affinity for DNA in NCP appears common since the reaction of many electrophiles including benzo[a]pyrene diol epoxide, nitrogen mustards, and mitomycin C are all suppressed rather uniformly by NCP assembly.^{3,5,7,8}

The efficiency of DNA alkylation by bisQM is mainly controlled by intercalation of its attached acridine,^{29,37} and thus, limiting this mode of binding would contribute to its impaired ability to target helical DNA. Intercalation of both ethidium bromide and aflatoxin B1 has already been reported to weaken for DNA packaged in a NCP versus free in solution and a similar effect likely dominates the response of bisQM.^{10,38} Such a suppression of reaction throughout the NCP would not necessarily have been expected for bisQM since intercalation also has the potential to localize near the dyad axis of NCP. For example, N-(2,3-epoxypropyl)-1,8-naphthalimide reacts selectively near the dyad axis where local distortion of the duplex structure may facilitate its intercalation.¹² The differences between these various intercalators may reflect differences in the orientation of the reactive groups when bound to DNA or differences in the specific nucleotide sequences used to assemble each model NCP.

BisQM Forms DNA–Protein Cross-Links in NCP.

Assembly of the NCP simultaneously demonstrated a protective function by suppressing reaction of the 601 DNA with bisQM and a harmful activity by fostering protein–DNA cross-linking. A broad range of cross-links was likely generated from incubation of the NCP with bisQM as evident from the diverse range of low mobility materials observed by electrophoresis under denaturing conditions (Figure 2). Cross-linking was only moderately efficient (~20%) despite the intimate association of histones and DNA within the NCP.

When compared to earlier reports, this yield was actually rather substantial. Previous studies with SV40 chromosomes demonstrated platinum-dependent cross-linking of DNA with many proteins but only minimal participation by histones.³⁹ More recently, cisplatin was shown to cross-link DNA to over 250 nuclear proteins *in vivo* but again the role of the histones was minor.⁴⁰ Likewise, nitrogen mustards and diepoxybutane generate over 130 DNA–protein cross-links and few contained the core histones.^{15,41} The low yields of such cross-linking may be initially surprising based on the multitude of possible interactions between the DNA and core histones, but certainly the lack of reaction is highly beneficial for minimizing the accumulation of lesions in cells.

DNA–protein cross-linking by bisQM is most likely the result of direct nucleophilic addition to its two electrophilic sites since the rate of this reaction is comparable to the rates of DNA alkylation. An alternative method of cross-linking is also possible indirectly from alkylation of guanine N7 even when generated by only monofunctional electrophiles. This modification promotes depurination to form abasic sites that readily conjugate with the side chain of proximal lysine residues.⁴² Such cross-linking has been observed in NCP with lysine residues from the N-terminal tails,^{42,43} but nucleosome assembly significantly suppresses depurination of N7 alkylated guanine relative to that in duplex DNA free in solution.⁴⁴ Cross-linking may additionally form by nucleophilic reaction at guanine C8 subsequent to modification at its N7.⁴⁴ However, many days are required to observe these types of cross-linking at 37 °C,⁴⁴ while the effects of bisQM are observed within 24 h of reaction at 4 °C (Figure 2).

QM Alkylation of Histones.

Protein–DNA cross-linking in the NCP may also be considered relatively efficient for bisQM when compared to how few adducts were detected for reaction of this electrophile with the histone octamer alone. Only two stable adducts were observed by UPLC–MS after tryptic digestion (Table 1). The monofunctional monoQM generated only slightly more adducts under equivalent conditions. Both *para*- and *ortho*-QMs were previously shown to react preferentially with the highly nucleophilic thiol of cysteine.^{45,46} Only a single cysteine is present in the model octamer of this investigation, and its lack of reaction can be ascribed to its inaccessibility. The next most reactive sites are the α -amines followed by the side chain amines (lysine).^{45,46} The lysine rich N-termini of the histones were consequently an expected target of mono- and bisQM, but no evidence of reaction at these positions was observed (Table 1). Sequestration of these termini by the helical DNA may account for their lack of reaction within the NCP, although alkylation also appeared minimal for the histone octamer free in solution. Alternatively, adducts of the lysine-rich tails may be too polar for resolution during reverse-phase chromatography or too labile for detection by UPLC–MS conditions. Such issues have been noted previously in studies of related *para*-QMs.⁴⁷ Still, the behavior of these histones stands in stark contrast to numerous other proteins (>300) that have been identified to form DNA conjugates with the *para*-QMs *in vivo*.^{47,48}

The *ortho*-QMs are generally considered more reactive than their *para*-QM isomers,⁴⁵ and the half-life of the simplest *ortho*-QM (6-methylene-2,4-cyclohexadien-1-one) has been measured under aqueous conditions to be ~0.14 s.⁴⁹ The half-life of the comparable *para*-

QM (4-methylene-2,5-cyclo-hexadiene-1-one) is almost 37-fold longer (~5 s).⁵⁰ Many nucleophiles have the potential to react with the transient mono- and bisQMs generated after deprotection of their precursors (Scheme 1). Nucleophiles such as water couple to these electrophiles relatively slowly, but their products form irreversibly. Comparable additions of thiols are most rapid but may only be reversed by heat.⁴⁶ Reaction with primary amines has not generally been considered reversible but may be sensitive to collision-induced dissociation during MS that could obscure their formation. QM fragments were detected from the MS² of certain regions in the chromatograms (Figures 3 and S5), but their origin could not be definitely assigned since no signal for the unmodified parent peptide was observed at a retention time that was different from its potential QM adduct. This result would only have been possible in the unlikely event of full conversion of the peptide to its QM conjugate.

Assigning the exact site of QM alkylation to a specific residue within the peptide conjugates summarized in Table 1 was not possible due to the dominant loss of the QM in the MS² analysis (Figures 3 and S4). No standard b and y ions created by backbone fragmentation were detected. The most nucleophilic site within the peptides of entries I, III, and IV is serine, and the only side chain possible in entry II is arginine. Neither seem very plausible due to their very low nucleophilicity under neutral conditions, and the site of coupling in the peptide of entry V is even more confounding. The carboxylate side chain may act as a nucleophile, but the resulting QM adduct is typically quite labile as illustrated from their use in the QM precursors, mono- and bisQMP (Scheme 1). Alternatively, the QMs could couple with the lysines and arginines at the C-termini of the peptides. This is typically expected to block trypsin digestion although exceptions have been reported.⁵¹ Most likely, the specificity reflects proximity of the side chains to hydrophobic binding sites within the histone octamer that associate with the nonpolar aromatic intercalator. Just as this component controls the reaction efficiency of DNA, so too may it control reaction of the histones (Figure 5).^{29,37} The lack of shared targets for the mono- and bisQM may simply highlight an exquisite sensitivity to orientation of the reactants. The inability to detect peptide conjugates attached to both benzylic positions of bisQM might be due to an instability of the resulting products under the analytical conditions but more likely reflects a lack of two nucleophiles that are both positioned appropriately to overcome competitive quenching by ubiquitous water. In general support of this latter explanation, a nitrogen mustard has been shown to yield monofunctional adducts in chromatin five-fold more readily than in DNA free in solution.⁵²

Reversibility of QM Reaction and NCP Assembly.

The reversible alkylation and regeneration of bisQM were first identified from reactions of nucleotides and DNA.^{30,35} This property prolonged the effective lifetime of the electrophilic intermediate and allowed complementary strands of DNA to exchange within cross-linked duplexes. A similar reversibility of the mono- and bisQM has now been shown to support their transfer from DNA to the histone octamer (Figure S8). The histones may therefore act in a sacrificial role as a terminal acceptor of the QM and essentially function to repair the DNA adducts. Assembly of the NCP consequently protects its DNA in two ways. First, its structure and environment suppress direct reaction of the QMs, and second, adducts formed at sites of reversible reaction (e.g., guanine N7) may transfer to irreversible sites on the

histones. At least from the results to date, modification of the histone octamer by bisQM did not interfere with subsequent assembly of the NCP (Figure 4B). The consequences of modification likely vary with structure and location since a lipid peroxidation product, 4-oxo-2-nonenal that conjugates to histidines and lysines, prevents nucleosome assembly when histones H3 and H4 are modified.¹³ Electrophilic modification of the lysine tails of NCP also has the potential to perturb the epigenetic regulations of genes,^{53,54} but no such modification by QM has yet been detected. Instead, QM alkylation of DNA suppresses NCP assembly (Figure 4A). Thus, the greatest impact of bisQM on chromatin is likely in the linker regions that do not benefit from the protective functions of the NCP. The resulting DNA adducts formed in these regions will likely interfere with chromatin remodeling by resisting later assembly into a NCP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

BisQM alkylation of DNA in the absence and presence of the histone octamer and after assembled into a NCP. Samples were treated with increasing concentrations of bisQM (0, 5, 50, 100, 250, 500 μ M) at 4 °C for 24 h and then treated with piperidine to identify sites of alkylation. The resulting fragments were separated by denaturing gel electrophoresis (10% PAGE) and visualized by phosphorimager.

Figure 2.

Formation of DNA-histone cross-links by treating the NCP with bisQMP. Individual samples of the NCP were treated with the indicated concentrations of bisQMP at 4 °C and quenched after 24 h by freezing in liquid N₂ as described in the Materials and Methods section. Samples were then thawed for 5 min at room temperature and divided into two sets. One set was analyzed directly, and the second set was treated with proteinase K (2 μ L, 1.6 U) for 15 min at room temperature. All samples were then combined with loading dye, separated by 10% denaturing SDS-PAGE, and visualized by phosphorimager.

Figure 3.

Peptide-QM adducts formed by the histone octamer. UPLC-MS analysis of the tryptic peptides formed by the histone octamer after treatment with (A) monoQMP and (B) bisQMP. Total ion count was monitored during elution of the peptides before (black) and after (red) reaction with the QMs. Signals unique to the alkylated samples are labeled according to Table 1. Signals labeled with "0" do not contain $[M + H]^+$ values that correspond to a QM adduct or peptide fragment. Peptide coverage by this analysis is summarized in Figure S3. (C) MS² spectrum of the adduct formed between monoQM and

TESSK of histone H2A (Table 1, entry I). (D) MS² spectrum of the adduct formed between bisQM and SAK of histone H2A (Table 1, entry IV).

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Figure 4. Reconstitution of NCP after alternative treatment of (A) 601 DNA and (B) histone octamer with bisQM. Lanes labeled as DNA contain DNA prior to reconstitution and lanes labeled NCP contain the products generated after reconstitution with the histone octamer. The relative distribution of species separated by native PAGE (6%) was measured by phosphorimaging the $[^{32}\text{P}]$ -labeled DNA.

Figure 5.
Location of protein adducts formed in NCP (PDB: 1KX5) from alkylation by monoQM and bisQM.

Scheme 1.

(A) General Strategy for Fluoride-Induced Generation of QM and Its Subsequent Alkylation of Nucleophile (Nuc); (B) *tert*-Butyl dimethylsilyl-Protected (TBDMS) Quinone Methide Precursors (MonoQMP and BisQMP) Used To Generate Electrophilic MonoQM and BisQM, Respectively

Scheme 2.
Detecting QM Transfer from DNA to Protein

Table 1.

QM-Peptide Adducts Observed from Alkylation of Histone Octamer and NCP

entry ^a	peptide-QM adduct	location of modified peptide	reaction with	experimental mass (Da)	calculated mass (Da) ^a	observed with NCP?	formed by QM transfer?
I	TESSK	H2A 121–125	monoQM	948.3704	948.4460	yes	yes
II	RR	either/and H2B 30–31, H3 53–54, H3 129–130, H4 46–47, H4 50–51	monoQM	728.2477	728.3990	no	no
III	IAGEASR	H2B 74–80	monoQM	1100.3669	1100.5522	no	yes
IV	SAK	H2A 124–129	bisQM	715.1870	715.3615	no	yes
V	DIQLAR	H3 126–128	bisQM	1126.3824	1126.3734	no	no

^aEntry numbers correlate to the signals indicated in the chromatograms of Figures 3 and S5.